libraries of longer or more diverse peptides should they be required for any given application.

We have expanded the applications of our peptide library approach by modifying the synthesis procedure to incorporate cleavable linkers on each bead. After exposure to the cleaving agent, such beads can then release a portion of their peptides into solution for biological assay while still retaining sufficient peptides on the beads for subsequent structure determination.

The one-bead, one-peptide concept and its applications discussed above demonstrate that this approach provides important new tools with which to search for specific ligands of potential diagnostic or therapeutic value. Such information should also enhance fundamental understanding of interactions between ligands and acceptor molecules. □

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Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery

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EXISTING methods for the synthesis and screening of large numbers of peptides are limited by their inability to generate and screen the requisite number (millions) of individual peptides[1–4] and/or their inability to generate unmodified free peptides in quantities able to interact in solution[4–8]. We have circumvented these limitations by developing synthetic peptide combinatorial libraries composed of mixtures of free peptides in quantities which can be used directly in virtually all existing assay systems. The screening of these heterogeneous libraries, along with an iterative selection and synthesis process, permits the systematic identification of optimal peptide ligands. Starting with a library composed of more than 34 million hexa-peptides, we present here the precise identification of an antigenic determinant recognized by a monoclonal antibody as well as the straightforward development of new potent antimicrobial peptides.

The initial synthetic peptide combinatorial library (SPCL) prepared and used in this work consisted of six-residue peptide sequences with acetylated N terminals and amidated C terminals. The first two positions in each peptide were individually and specifically defined, whereas the last four positions consisted of equimolar mixtures of 18 of the 20 natural L-amino acids (for ease of synthesis, cysteine and tryptophan were omitted in this initial library). Such libraries can be generally represented by the sequence Ac-O_1O_2XXXX-NH_2 (where Ac represents acetyl) (see legend to Fig. 1).

Using a competitive enzyme-linked immunosorbent assay (ELISA), each of the 324 different peptide mixtures of the SPCL (Ac-O_1O_2XXXX-NH_2) was assayed to determine its ability to inhibit the interaction of a monoclonal antibody with a larger 13-residue peptide (Ac-YPYDVPDYASLRS-NH_2; single-letter amino-acid code). Of the 324 peptide mixtures examined (Fig. 1), Ac-DVXXXX-NH_2 caused the greatest inhibition of antibody binding (Table 1). Twenty new peptide mixtures were then synthesized in which the third position of the peptide mixture Ac-DVXXXX-NH_2 was defined (Ac-DVOXXX-NH_2, tryptophan now included in the X positions). Each new peptide mixture contained 6,859 (19^3) individual peptides (137,180 in total). The most effective inhibiting peptide mixture was Ac-DVPXXX-NH_2 (50% inhibitory concentration, $IC_{50} = 41$ μM; Table 1*b*). The above iterative process, which reduces the number of peptide sequences by 20-fold each time it is repeated, was then carried out for the remaining three positions (Table 1, *c–e*). It should be noted that on defining the fifth position (Ac-DVPDOX-NH_2, Table 1*d*), the IC_{50} found for Ac-DVPDYX-NH_2 (0.38 μM) was at least 3,500-fold lower than any of the other 19 peptide mixtures. Also, the peptide mixtures Ac-DVPDXX-NH_2 and Ac-DVPXXX-NH_2 had IC_{50} values lower than all of the peptide mixtures with the fifth position defined, with the exception of Ac-DVPDYX-NH_2. This clearly

TABLE 1 Identification of the antigenic determinant recognized by monoclonal antibody 19B10

Peptide mixture	IC_{50} (μM)	Peptide	IC_{50} (μM)
(*a*)		(*e*)	
Ac-DVXXXX-NH_2	250	Ac-DVPDYA-NH_2	0.03
Ac-DIXXXX-NH_2	318	Ac-DVPDYS-NH_2	0.27
Ac-DMXXXX-NH_2	752	**Ac-DVPDYX-NH_2**	**0.38**
Ac-DLXXXX-NH_2	>1,400	Ac-DVPDYC-NH_2	0.90
		Ac-DVPDYV-NH_2	1.10
(*b*)		Ac-DVPDYT-NH_2	1.50
Ac-DVPXXX-NH_2	41	Ac-DVPDYG-NH_2	1.60
Ac-DVEXXX-NH_2	146	Ac-DVPDYE-NH_2	4.06
Ac-DVQXXX-NH_2	215	Ac-DVPDYI-NH_2	5.29
Ac-DVXXXX-NH_2	**250**	Ac-DVPDYM-NH_2	7.70
Ac-DVMXXX-NH_2	451	Ac-DVPDYQ-NH_2	8.18
Ac-DVRXXX-NH_2	906	Ac-DVPDYH-NH_2	8.99
Ac-DVAXXX-NH_2	1,107	Ac-DVPDYL-NH_2	9.98
Ac-DVCXXX-NH_2	>1,400	Ac-DVPDYR-NH_2	10.90
		Ac-DVPDYF-NH_2	12.02
(*c*)		Ac-DVPDYN-NH_2	15.56
Ac-DVPDXX-NH_2	4.4	Ac-DVPDYK-NH_2	17.60
Ac-DVPXXX-NH_2	**41**	Ac-DVPDYY-NH_2	22.48
Ac-DVPAXX-NH_2	>1,400	Ac-DVPDYP-NH_2	26.14
		Ac-DVPDYW-NH_2	32.14
(*d*)		Ac-DVPDYD-NH_2	48.00
Ac-DVPDYX-NH_2	0.38		
Ac-DVPDXX-NH_2	**4.4**		
Ac-DVPDAX-NH_2	>1,400		

The IC_{50}s of the most effective inhibitory peptide mixtures obtained at each iterative step are illustrated for: *a*, peptide mixtures from the initial screening of the SPCL; *b*, the third position defined (AC-DVOXXX-NH_2); *c*, the fourth position defined (Ac-DVPOXX-NH_2); *d*, the fifth position defined (Ac-DVPDOX-NH_2); and *e*, the sixth position defined (Ac-DVPDYO-NH_2). The IC_{50} of the peptide mixture derived from the previous iterative step is in bold for comparison. Peptide mixtures were assayed by competitive ELISA (see Fig. 1). The concentration of each peptide mixture necessary to inhibit 50% of the antibody binding to the control peptide on the plate was obtained by serial dilutions of the peptide mixture. The IC_{50}s were calculated using the software GRAPHPAD (ISI, San Diego). The four-step iterative screening and synthesis process takes approximately 4 weeks. This time frame will vary depending on the assay being used and the number of cases moved forward at each iterative step.

illustrates the importance of the presence of specific peptide sequences in each peptide mixture. Among the 20 peptides in which the sixth position was defined, Ac-DVPDYA-NH_2 had the lowest IC_{50} (0.03 μM; Table 1*e*). This sequence exactly matches the antigenic determinant found in earlier studies to be recognized by this monoclonal antibody[3,9,10]. With other screening library procedures[6-8], such precise sequence determination, or the identification of different sequences with affinities equal to or exceeding existing sequences, was not accomplished. The results presented here confirm our earlier work in which individual peptides[9-11] or chemically synthesized heterogeneous peptide mixtures[12,13] were used to establish that each position in a linear antigenic determinant has a specific, quantifiable rank order of importance. The use of this SPCL permits the ready determination of the specific peptide sequence that bound to this antibody out of a total of 34,012,224 possible hexapeptides. Note that no information about the sequence of the antigen or antibody is required to carry out determinations of this kind.

The development of new, potentially useful therapeutic peptides[14-19] requires the synthesis and screening of hundreds to thousands of analogues of an original, often serendipitously discovered active sequence. The potential of SPCLs for the development of new antimicrobial peptides against *Staphylococcus aureus* (Gram-positive bacteria), *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative bacteria), and the yeast *Candida albicans* was examined in microdilution assays using the same set of 324 peptide mixtures making up the Ac-O_1O_2XXXX-NH_2 peptide library used above. Although many antimicrobial lead peptide sequences derived from this SPCL have been followed in detail (manuscript in preparation), a single example (Ac-RRXXXX-NH_2) found effective against the above microorganisms is presented here. Positions three to six of Ac-RRXXXX-NH_2 were defined using the iterative process described above (the data for *S. aureus* are shown in Table 2). The minimum inhibitory concentrations (MIC) of the 20 individual peptides obtained on defining the sixth position of Ac-RRWWCX-NH_2, as well as the C-terminal amide form of the naturally occurring antimicrobial peptide magainin, are shown in Table 3. The hexa-peptide Ac-RRWWCR-NH_2 was the most active of this set. Its MIC against *S. aureus* was 3.2–6.5 μg ml^{-1}. Preliminary data indicate that this peptide is bacteriocidal. The haemolytic activity of Ac-RRWWCR-NH_2 was less than 0.2% at 500 μg ml^{-1}. It is noteworthy that the antistaphylococcal activities of 17 of the 20 sequences were greater than magainin[15].

The use of SPCLs has been illustrated here for both the precise identification of a linear antigenic determinant recognized by a monoclonal antibody and for the development of new, highly effective antimicrobial peptides. A number of other libraries,

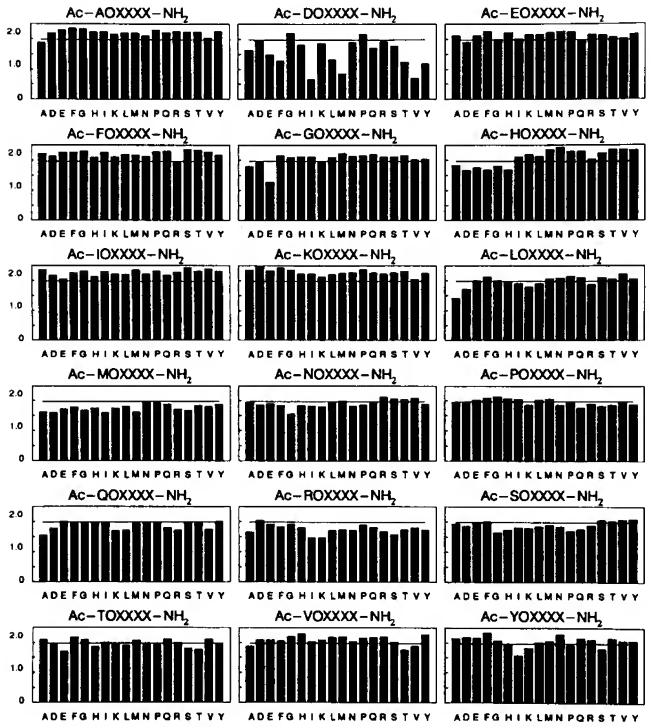

FIG. 1 Initial screening of the SPCL (Ac-O_1O_2XXXX-NH_2) for ability to inhibit the binding of monoclonal antibody 19B10. Each of the 324 peptide mixtures of the SPCL was assayed by competitive ELISA for its ability to inhibit the binding of monoclonal antibody 19B10 (ref. 20) to the plate-adsorbed peptide Ac-YPYDVPDYASLRS-NH_2. The individual bar graphs are segregated by first amino acid (O_1), with the individual bars in each graph representing the 18 individual amino acids making up the second position (O_2). The *y*-axis represents optical density (OD) at 492 nm. The horizontal line in each bar graph represents the average OD of the 324 peptide mixtures. O_1 and O_2 are specific individual amino acids; that is O_1O_2=AA, AD, AE, and so on through to YV, YY, for a total of 324 combinations at positions O_1 and O_2 (18^2). Each X position represents an equimolar mixture of the 18 amino acids A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, Y for a total of 104,976 combinations (18^4). Each of the 324 different peptide mixtures consists of 104,976 individual hexamers, which represent 34,012,224 peptides in total (324×104,976). Assuming an average relative molecular mass for Ac-O_1O_2XXXX-NH_2 of 785, then a mixture of 104,976 peptides (18^4) at a total final concentration of 1.0 mg ml^{-1} yields a concentration of every peptide in each mixture of 9.53 ng ml^{-1} (12.1 nmol l^{-1}).

METHODS. The synthetic peptide library was prepared using methylbenzhydrylamine (MBHA) polystyrene resin and standard *t*-Boc chemistry in combination with simultaneous multiple peptide synthesis (SMPS[3]). A divide, couple and recombine (DCR) process was used to synthesize the XXXX-peptide resin. This process assures equimolarity of the peptides on the resin. Briefly, 18 porous polypropylene packets, each containing 4.65 mmol (5.00 g) of MBHA resin, were coupled to each of the protected *N*-α-*t*-Boc amino acids of interest. All coupling reactions proceeded to completion (>99.5%), as assessed by Gisin's picric acid[21] or Kaiser's ninhydrin tests[22]. The resulting resins from each packet were then combined and thoroughly mixed. This resin mixture was separated into 18 portions of equal weight which were placed into porous polypropylene packets, followed by *N*-α-*t*-Boc protecting group removal and neutralization of the resulting amine TFA salts. The resin packets were then reacted with solutions of the individual activated amino acids to yield the 324 dipeptide combinations (18^2). The above DCR process was repeated twice more, yielding a final mixture of 104,976 protected tetra-peptide resins (18^4). This XXXX-resin was divided into 324 aliquots (150 mg each) and placed in numbered, porous polypropylene packets. Synthesis of the next two defined positions was carried out by SMPS. The peptide mixtures were deprotected and cleaved from their respective resins using low–high hydrogen fluoride (HF)[23] as described for individual peptides earlier[17,24] in a multiple HF cleavage apparatus (Multiple Peptide Systems, San Diego, California). Extraction of the individual peptide mixtures was carried out with H_2O. The time frames for synthesis, and the amounts of each peptide mixture obtained, are the same as described earlier for this number of individual peptides[3]. The competitive ELISA used is a modification of the direct ELISA technique described previously[10]. It differs only in the antibody addition step in which 25 μl each peptide mixture of the SPCL was added, with a fixed dilution of antibody 19B10 (25 μl per well).

TABLE 2 Antimicrobial activity against *S. aureus* obtained in the iterative process

Peptide mixture	IC_{50} ($\mu g\ ml^{-1}$)	Peptide mixture	IC_{50} ($\mu g\ ml^{-1}$)
(*a*)		(*c*)	
Ac-RRWXXX-NH_2	216	Ac-RRWWCX-NH_2	8.7
Ac-RRYXXX-NH_2	239	Ac-RRWWWX-NH_2	9.9
Ac-RRRXXX-NH_2	275	Ac-RRWWRX-NH_2	9.9
Ac-RRHXXX-NH_2	286	Ac-RRWWFX-NH_2	12
Ac-RRCXXX-NH_2	338	Ac-RRWWIX-NH_2	14
Ac-RRXXXX-NH_2	**450**	**Ac-RRWWXX-NH_2**	**32**
(*b*)		(*d*)	
Ac-RRWWXX-NH_2	36	Ac-RRWWCR-NH_2	3.4
Ac-RRWFXX-NH_2	58	Ac-RRWWCW-NH_2	4.1
Ac-RRWRXX-NH_2	77	Ac-RRWWCV-NH_2	4.9
Ac-RRWCXX-NH_2	88	Ac-RRWWCY-NH_2	5.4
Ac-RRWLXX-NH_2	178	Ac-RRWWCK-NH_2	5.5
Ac-RRWXXX-NH_2	**273**	**Ac-RRWWCX-NH_2**	**8.7**

The five lowest IC_{50}s obtained are illustrated for the peptide mixtures on defining: *a*, the third position (Ac-RROXXX-NH_2); *b*, the fourth position (Ac-RRWOXX-NH_2); *c*, the fifth position (Ac-RRWWOX-NH_2); and *d*, the sixth position (Ac-RRWWCO-NH_2). The IC_{50} of the peptide mixture derived from the previous iterative step is in bold for comparison. The antimicrobial activity of each peptide mixture against *S. aureus* ATCC 29213 was determined as described earlier[19]. Briefly, in 96-well tissue culture plates, peptide mixtures were added to the bacterial suspension ($1–5 \times 10^5$ colony-forming units ml^{-1}) at concentrations derived from serial twofold dilutions ranging from 1.5 mg ml^{-1} to 2.9 $\mu g\ ml^{-1}$. The plates were incubated overnight at 37 °C, and the growth determined at each concentration by the optical density at 620 nm. The relative per cent of growth found for each set of peptide mixtures was consistent in three separate determinations. The IC_{50}s were then calculated using the software program GRAPHPAD (ISI).

TABLE 3 Antimicrobial activity of Ac-RRWWCO-NH_2 against *S. aureus*

Sequence	MIC ($\mu g\ ml^{-1}$)	Sequence	MIC ($\mu g\ ml^{-1}$)
Ac-RRWWCR-NH_2	3.2–6.5	Ac-RRWWCA-NH_2	9–18
Ac-RRWWCV-NH_2	3.8–7.7	Ac-RRWWCP-NH_2	10–19
Ac-RRWWCW-NH_2	4.5–9.0	Ac-RRWWCM-NH_2	14–27
Ac-RRWWCY-NH_2	4.7–9.5	Ac-RRWWCL-NH_2	14–27
Ac-RRWWCK-NH_2	4.8–9.6	Ac-RRWWCI-NH_2	17–34
Ac-RRWWCT-NH_2	4.9–10	Ac-RRWWCF-NH_2	17–34
Ac-RRWWCH-NH_2	5.5–11	Ac-RRWWCC-NH_2	19–38
Ac-RRWWCQ-NH_2	6–12	**Magainin-II-NH_2**	**32–64**
Ac-RRWWCS-NH_2	7–14	Ac-RRWWCE-NH_2	>250
Ac-RRWWCX-NH_2	**7–14**	Ac-RRWWCG-NH_2	>500
Ac-RRWWCN-NH_2	8–16	Ac-RRWWCD-NH_2	>1,000

The MICs for the 20 peptides in which the sixth position is defined (Ac-RRWWCO-NH_2) are shown. The MIC is defined as the lowest concentration of peptide at which no growth is detected after 21 h incubation at 37 °C. The MICs of the previous peptide mixture and magainin-II are bold for comparison.

such as one composed entirely of D-amino acids, have been prepared which in total permit the systematic screening of hundreds of millions of peptides. A fundamental feature of SPCLs is that free peptides can be generated and used in solution in virtually all existing assay systems at a concentration of each peptide most applicable to the assay. This approach has also been successfully used in radio-receptor assays (opioid peptides) and plaque inhibition assays (human immunodeficiency virus (HIV-1) and herpes simplex virus (HSV). SPCLs, as described, greatly aid all areas of drug discovery and research involving peptides. □

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ERRATUM

Ancient oceans, ice sheets and the hydrological cycle on Mars

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IN this Article in the 15 August 1991 issue, an error in the *Nature* office led to the omission of a line from Table 3. In addition, some corrections noted by the authors were not made before publication. Corrected versions of the relevant passages appear below and in reprints.

TABLE 3 Possible sources of CO_2 related to catastrophic outflow and ocean formation on Mars

Source	CO_2 partial pressure (mbar)
North polar cap	~20
Massive volcanism	~100
Adsorbed on regolith: ocean basin	≤100
land	≤350
Groundwater	≤1,300
CO_2 clathrate	≤4,000

Fluvial history

In first paragraph:

The very high infiltration capacities of common martian surface rocks (lava flows and impact-brecciated regolith) would allow subsurface aquifers to be replenished easily, so that head differentials could be sustained and drive prolonged groundwater flow. The resulting sapping[31] would then produce the observed pattern of structurally controlled, low-density valley networks[24].

Although Noachian valleys are consistent with atmospheric